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14. ABSTRACT

We have developed several novel small molecule inhibitors of BCR-ABL that inhibit the proliferation and induce apoptosis of CML cell lines that express the WT or the T315I mutant form of BCR-ABL. These compounds readily induced the down-regulation of BCR-ABL auto-phosphorylation and STAT-5 phosphorylation. Using ON044580 as the lead compound, we have carried out chemical modification of the compound to facilitate the oral bio-availability of the compound. This resulted in the derivation of ON045590 which retained the BCR-ABL inhibitory activity as well as JAK2 inhibitory activity of the parent compound. Caco-2 cell bioavailability assays suggest that this compound is likely to be orally bio-available. We show that ON044850 destroys the Bcr-Abl/Jak2 protein Network, which is a large multi-component signaling structure maintained in an active state by members of the HSP90 chaperone complex. ON044850 blocks JAK2-mediated Tyr705 phosphorylation of STAT3 as well as Tyr177 of BCR-ABL. Our results also show that ON044580 inhibits NF-kB activation mediated by JAK2.

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Introduction

The recent surge in the development of targeted kinase inhibitors as therapies for cancer was spurred from the success of imatinib (Gleevec®, STI571) for the treatment of Philadelphia chromosome-positive chronic meylogenous leukemia. This drug, for the first time, showed that a small molecule could be designed to inhibit an oncogenic tyrosine kinase (BCR-ABL) that was responsible for inducing malignant transformation of a particular cell type. In spite of the fact that the majority of patients receiving imatinib respond to treatment at both the hematological and cytogenetic levels, relapse occurs in a subset of patients with chronic disease, and this number jumps significantly to nearly 100% in those patients whose disease is in the advanced stages (reviewed in 1). Because of the frequency of mutations, it has become important to develop novel inhibitors that are active against imatinib resistant mutants of BCR-ABL. In response to this demand, two different promising compounds have recently been approved for the treatment of CML. These compounds known as BMS-354825 or Dasatinib, (2) and AMN107 or Nilotinib (3) were found to be inhibitory to nearly all imatinib-resistant forms of BCR-ABL with the exception of one mutation at position 315 where Threonine is replaced by Isoleucine (T315I). As this particular mutation is the most resistant to imatinib and emerges in the largest percentage of patients who develop resistance (reviewed in 4), there is an urgency to develop alternative compounds that are capable of inhibiting this particular (as well as other) amino acid substitution. A major goal of our team was to generate a potent inhibitor of BCR-ABL by targeting regions outside the ATP binding site of this enzyme as these compounds offer the potential to be unaffected by mutations that make CML cells resistant to imatinib.

Body

Work accomplished by Dr. Reddy's Group

In last year's report we described the synthesis of 13 modifications of ON044580 on the benzoyl ring to enhance aqueous solubility and bioavailability of ON044580. Of these ON044690 and ON045260 were found to effectively inhibit wild-type BCR-ABL and T315I-BCR-ABL in cells and moderately inhibit their biochemical kinase activities *in vitro*. However, only ON045260 retained JAK-2 inhibitory activity comparable to the parent compound. This year's report describes bioavaiability studies with these

modifications of ON044580, the development of ON045590 as an intermediate for lead optimization based on structure activity relationship (SAR) analysis and the simultaneous development of a nano-particle delivery system for ON044580 to overcome toxicity and solubility issues.

Caco-2 Cell Bioavailablity studies

Caco-2 cell permeability assays were performed on these modifications of ON044580 to test their bioavailability. Of the 13 compounds tested only 5 showed moderate to excellent Caco-2 cell permeability: ON044790, ON044870, ON045050, ON045230 and ON045240. However, these derivatives of ON044580 lost their cytotoxic and kinase inhibitory activities. Conversely, the modifications that retained biological activity, including ON045260, exhibited poor transport across the cells, indicating these modifications on the benzoyl ring did not improve the bioavailability of these molecules. This work is summarized in Table 1 which shows cytotoxicity data against three cell lines (K562, Ba/F3:V617F-*JAK2* and DU145) in addition to cell permeability for the compounds discussed.

Table 1: In vitro cytotoxicity of ON044580 derivatives:

Compound	**K562 (µM)**	**Ba/F3:V617F-*JAK2* (µM)**	**DU145 (µM)**	**Caco-2 permeability**
ON044790	5.0	>10	20	+
ON044870	7.5	>10	5.0	+
ON044970	0.6	0.7	0.2	-
ON045000	0.2	1.3	1.5	-
ON045050	0.75	1.3	3.0	-
ON045230	1.0	>10	4.0	+
ON045240	1.0	>10	5.0	+
ON045260	0.3	0.4	2.0	-
ON045480	7.5	>10	15	+

Modifications on the benzyl aromatic ring of ON044580

The above structure activity relationship (SAR) studies indicate that bioavailability of ON044580 can be improved by replacing one of the non-essential groups or atoms on one of its aromatic rings. Since the modifications on two of the three rings did not produce

the optimized molecule, our informed strategy is to modify the groups that are present on the benzyl aromatic ring. In order to attach bioavailable groups (secondary and tertiary amines) it is necessary to make a intermediate with a tosyl moiety on the benzyl aromatic ring. We made this modification and called the resultant intermediate ON045590 whose structure is shown in Figure 1.

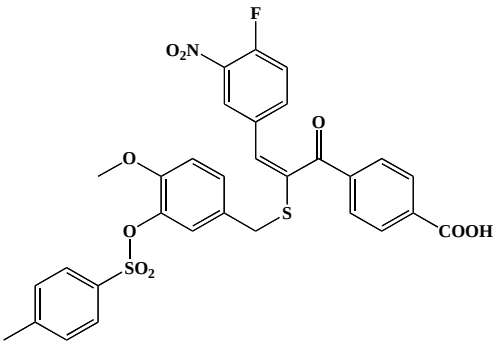


Figure 1: Structure of ON045590

Having obtained the intermediate ON045590, we next assessed its activity in biological assays and found that the addition of a tosyl ester has not altered its cytotoxicity profile or its biological activity.

BCR-ABL kinase inhibitory activity of the intermediate ON045590

We tested the ability of ON045590 to inhibit the *in vitro* kinase activities of BCR-ABL proteins immunoprecipitated from mammalian cells. For these studies, cell lysates were prepared from K562 cells expressing the wild type BCR-ABL or 32D cells expressing the T315I mutant form of BCR-ABL. The immunoprecipitates were washed, re-suspended in kinase buffer and used for kinase assays using the GST-Abltide as a substrate. Immunoprecipitates derived from 200 µg of total protein were used for a single assay reaction and were mixed with different concentrations of the inhibitor. The kinase assays were performed as described previously by us. Imatinib was used as a control in all of these assays.

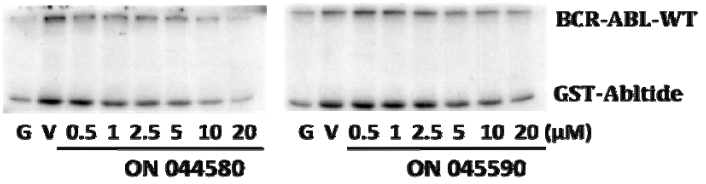


Figure 2: Inhibition of wild type BCR-ABL kinase activity by ON044590. 200μg protein from lysates of K562 cells expressing wild-type BCR-ABL were incubated with anti BCR (N-20) antibody and immunoprecipitates were prepared using protein G sepharose beads. The immunoconjugates were treated with the indicated concentrations of ON045590, ON044580 or vehicle (V) or Gleevec (G; 1μM) for 30 minutes and processed for radiometirc kinase assays in the presence of 20μM ATP, 40 μci Ɣ-^{32}P-ATP and 1 μg GST-Abltide as substrate for 20 minutes at 30°C. The sampels were resolved by 10 % SDS-PAGE and autoradiographed.

As shown in Figure 2, ON044580 potently inhibited the autophosphorylation of WT BCR-ABL and phosphorylation of GST-Abltide while the inhibitory activity of ON045590 was only slightly lower. As expected, imatinib inhibited both phosphorylations at 1μM.

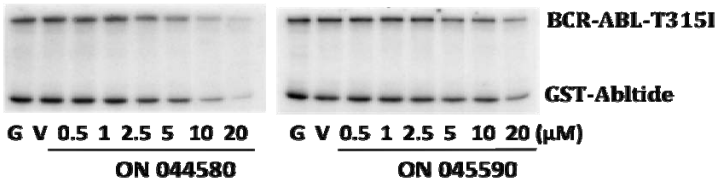


Figure 3: Inhibition of Gleevec resistant T315I-BCR-ABL kinase activity by ON044590. 200μg protein from lysates of 32D:*p210*-T315I cells expressing T315I mutant of BCR-ABL were incubated with anti BCR (N-20) antibody and immunoprecipitates were prepared using protein G sepharose beads. The immunoconjugates were treated with the indicated concentrations of ON045590, ON044580 or vehicle (V) or Gleevec (G; 10μM) for 30 minutes and processed for radiometirc kinase assays in the presence of 20μM ATP, 40 μci Ɣ-^{32}P-ATP and 1 μg GST-Abltide as substrate for 20 minutes at 30°C. The sampels were resolved by 10 % SDS-PAGE and autoradiographed.

We next compared the inhibitory activity of these compounds against the imatinib resistant T315I mutant form of BCR-ABL. Figure 3 shows that while imatinib failed to inhibit the mutant kinase at 10μM, ON044580 inhibited its autophosphorylation very potently. ON045590 by comparison was moderately active against the imatinib resistant form of BCR-ABL.

Imatinib inhibits only the WT form of BCR-ABL. In contrast ON045590 inhibits both WT and T315I mutant forms of BCR-ABL kinase, suggesting that mutations that affect

the kinase inhibitory activity of imatinib do not affect the inhibitory activity of this modification of ON044580.

***In vivo* inhibition of BCR-ABL phosphorylation and signaling by ON045590.**

To test the *in vivo* wild-type BCR-ABL inhibitory activity of ON04550 we treated K562 cells with increasing concentrations of the compounds for 2 hrs. Cells were washed and lysed in detergent containing buffer and the clarified lysates subjected to SDS-PAGE followed by western blotting to detect the phosphorylation status of BCR-ABL and STAT-3. The results of this study, presented in Figure 4, show that ON045590 is able to inhibit the phosphorylation of BCR-ABL and its downstream target STAT-3 in a concentration dependent manner.

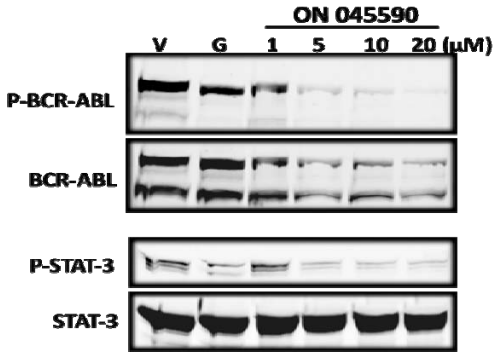


Figure 4: Inhibition of BCR-ABL pathway by ON044590 in K562 cells. K562 cells expressing wild-type BCR-ABL were treated with the indicated concentrations of ON045590 or vehicle (V) or Gleevec (G; 1μM) for 2 hours. The cells were harvested and total cellular lysates were prepared. Equal amounts of proteins was resolved by 10 % SDS-PAGE and analyzed for indicated proteins by infrared labeled secondary antibodies and scanning with Odyssey scanner (LiCor Technology).

***In vitro* tumor cell killing activity of ON045590**

We next examined the ability of ON045590 to inhibit the proliferation of BCR-ABL positive myeloid leukemias. To this end we studied the ability of this compound to inhibit the growth of imatinib sensitive K562 cells which express WT BCR-ABL kinase and used the human prostrate cancer cell line DU145 as a comparison. The results presented in Figure 5 show ON045590 is an effective inducer of tumor cell death exhibiting GI_{50} values of 4 μM for K562 cells and 1.5 μM for DU145 cells. This seems to be a reversal of trend from ON044580 which showed better activity against K562 as compared to DU145 cells.

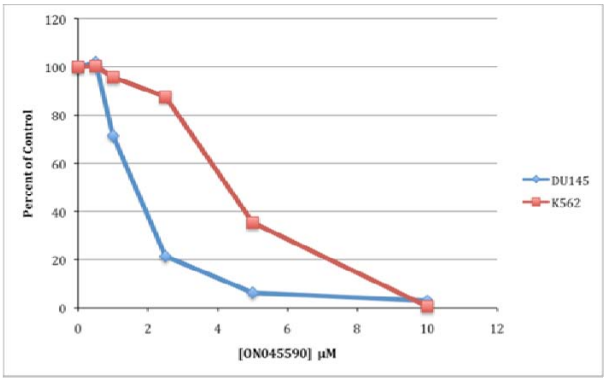


Figure 5: ON044590 inhibits the growth of tumor cell lines. K562 cells were plated at 2.5 x 10^5 cells/ml/well. The cells were treated with increasing concentrations of ON045590 and the total number of viable cells was determined following trypan blue staining and counting using a haemocytometer 96 hours later. The data is plotted as the percent total viable cells compared to DMSO treated controls.

JAK2 inhibitory activity of ON compounds

ON044580 is a dual inhibitor of BCR-ABL and JAK-2 kinases. Having confirmed that ON045590 inhibits BCR-ABL we were keen to learn of its ability to inhibit JAK-2. To test the JAK2 inhibitory activity of ON045590, we used the recombinant constitutively activated V617F mutant form of this protein produced in insect cells (which is commercially available). As shown in Figure 6 we found that ON045590 shows potent JAK-2 kinase inhibitory activity easily comparable to the parent molecule (ON044580).

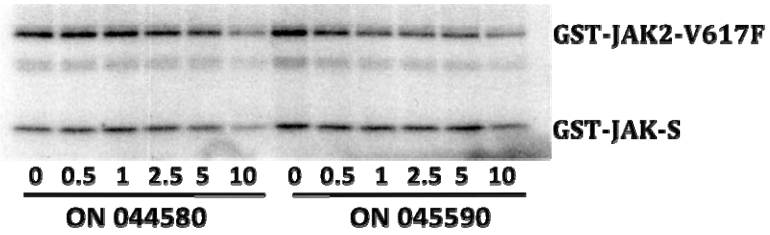


Figure 6: Inhibition of V617F-JAK2 kinase activity by ON044590. 200ng GST-V617F-JAK2 (aa 532-1132) was incubated the indicated concentrations of ON045590 and ON044580 for 30 minutes. The samples were then processed for radiometirc kinase assays in the presence of 20µM ATP, 40 µci Ɣ-^{32}P-ATP and 1 µg GST-*JAK*-S as substrate for 20 minutes at 30°C. The sampels were resolved by 10 % SDS-PAGE and autoradiographed.

Growth inhibition of V617F-*JAK2* expressing cells.

To determine whether ON045590 inhibits the proliferation of V617F-*JAK2*-positive leukemic cells as potently as ON044580, we studied its effect on the growth and viability of Ba/F3:V617F-*JAK2* cells which were transfected with an expression vector that encodes the mutant *JAK2*. The results of this study, presented in Figure 7 show that ON045590 can readily inhibit the proliferation and induce apoptosis of all this cell line with a GI_{50} value of 380 nM which matches 350 nM of ON044580 we reported earlier.

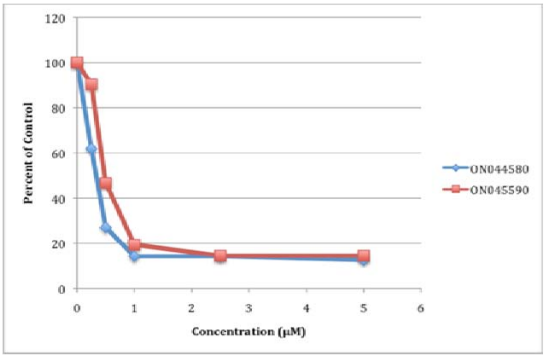


Figure 7: ON044590 inhibits the growth of Ba/F3:V617F-*JAK2* cells. Ba/F3:V617F-JAK2 cells were plated at 1.0 x 10^5 cells/ml/well. The cells were treated with increasing concentrations of ON044580 and ON045590. The total number of viable cells was determined following trypan blue staining and counting using a haemocytometer 72 hours later. The data is plotted as the percent total viable cells compared to DMSO treated controls.

Hence, ON045590 was found by us to be a suitable intermediate of ON044580 retaining its dual kinase inhibitory and cytotoxic properties. We are currently introducing groups containing secondary and tertiary amines with the hope that the resulting molecule will be more bioavailable than ON044580 and still retain its biological activities.

Nanoparticle delivery of ON044580

In our attempt to assess the efficacy of ON044580 in tumor xenografts models we first attempted to test its toxicity in mice. We found ON044580 dissolved in DMSO to be toxic by tail vein injection and subcutaneous administration at doses of 25mg/kg body weight. This toxicity appears to be partially to be due to DMSO which was used as a solvent. In addition, ON044580 was found to be very insoluble and biologically unavailable. Our attempts at modifying the available side groups of ON044580 invariably led to decrease in its potent kinase inhibitory and/or cytotoxic activities. Hence, we

decided to use targeted nanoparticles as a delivery system for administration of ON044580 and its bioactive analogs. We chose to utilize the transferrin-transferrin receptor system to encapsulate the drug because of the biological advantages that this system offers.

Metabolically active cells demand high amounts of iron. Rapidly proliferating malignant cells [5] and metabolically active brain cells [6] express higher number of transferrin receptors to facilitate higher iron transport. The use of apotransferrin, transferrin and transferrin receptor antibodies (OX-26) as carriers of drug for targeted delivery to transferrin receptor expressing cells is extensively studied [7].

To reduce the entry of drug into non target cells, several target specific technologies have been developed during the last few years and implemented. Most of these technologies invoke the use of polymeric materials (PEG, PLGA) [8] and proteins (chitins, albumin). These materials though excellent carriers of drug, lack target specificity [9]. Thus target specific ligands like transferrin, antibodies, carbohydrates, signal peptides are widely studied for guiding these delivery systems to specific cells and tissues [10]. We reports a target directed nanoparticle drug delivery system comprising of drug loaded transferrin nanoparticles.

Method of preparation of nanoparticles

The schematic below (Figure 8) details the method for preparation of transferring nanoparticles containing ON044580 and Doxorubicin as a control.

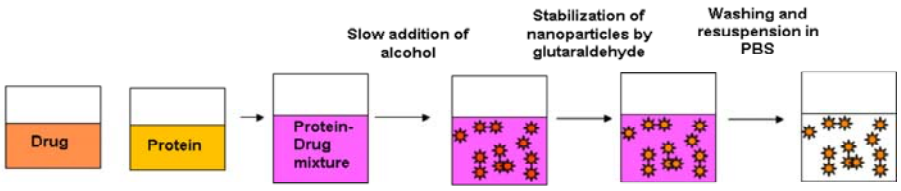
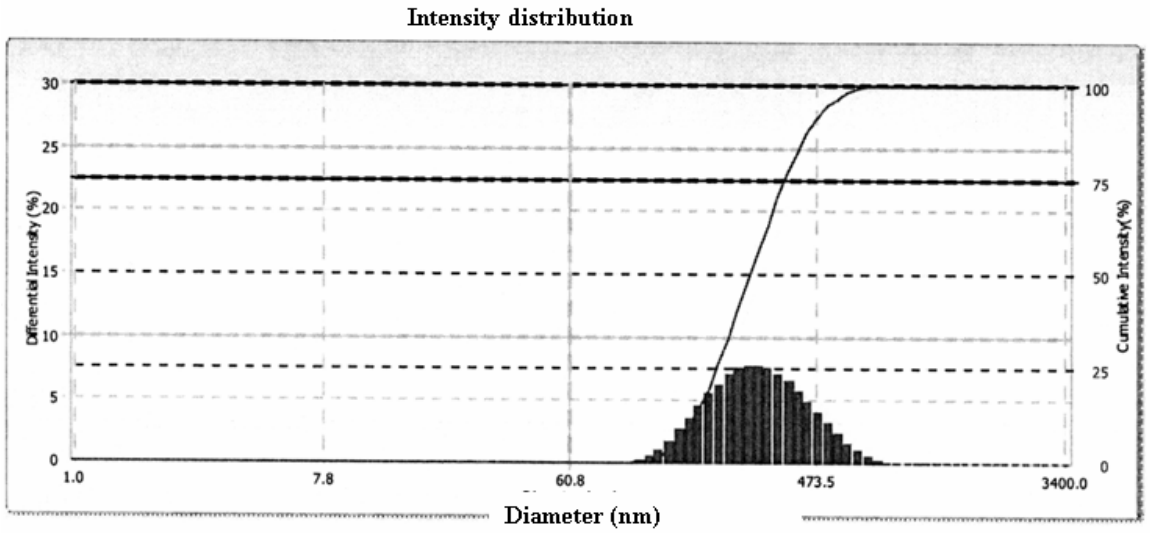


Figure 8: Preparation of drug containing Transferrin nanoparticles. 10 mg of human holotransferrin was dissolved in sterile PBS (800 µL) with gentle shaking and slow pippeting. 100 µL of DMSO/drug in DMSO was added and incubated at room temperature for 30 minutes. This was followed by addition of 600 µL absolute in 12 batches of 50 µL every 5 minutes with gentle rotation. 1 µL of glutaraldehyde was added and gently rotated for 30 minutes. The preparation was centrifuged 4000 x g for 10 minutes, the supernatant was discarded and the pellet dispersed in sterile PBS. This washing step was repeated twice to remove unbound drug and the final pellet was dispersed in 1 mL of PBS and used for further analysis.

Size of nanoparticles

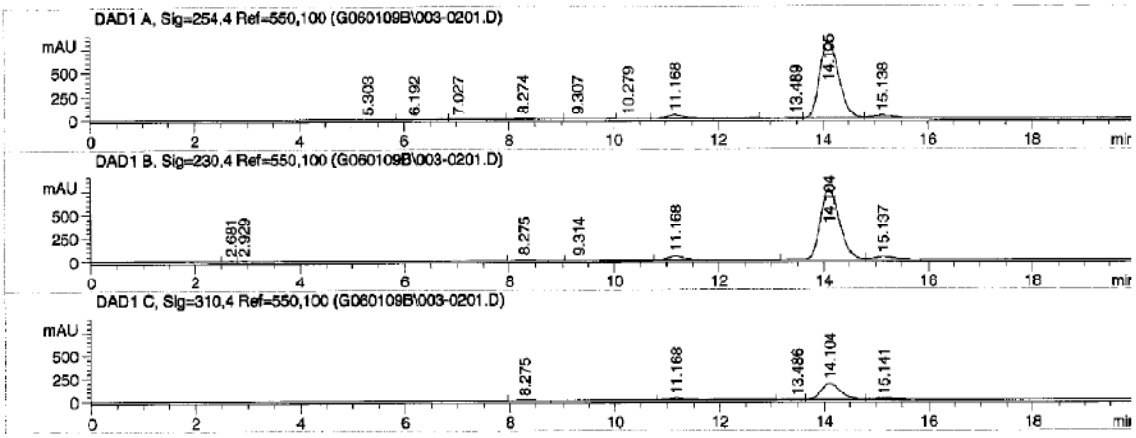
The size of the particles was measured by the Beckmann Delsa Nano instrument. The average diameter of drug loaded transferrin nanoparticle was found to be 307nm with a standard deviation of 122nm using the zeta potential sizing method. As is evident from the chart below, the majority of nanoparticles were well below 500nm in diameter.



Estimation of drug encapsulation in nanoparticles

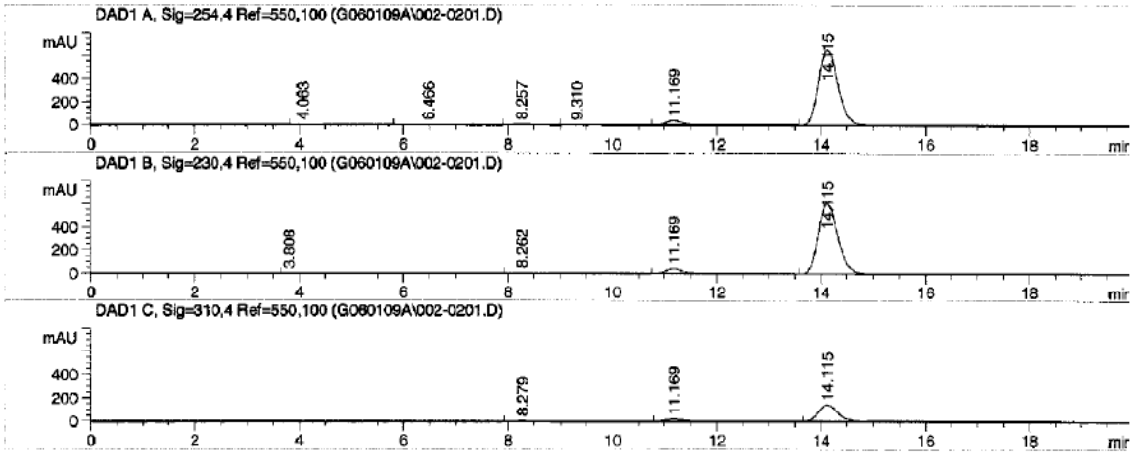
In order to be used in biological assays it is imperative that the concentration of drug within the nanoparticle preparation be determined. To that end, 100 µL of the drug-loaded nanoparticles were centrifuged at 4000 x g and the supernatant discarded. The pellet was dispersed in 300 µL acetonitrile and vortexed for 10 minute to release the drug from the nanoparticles. The mixture was centrifuged at 12,000 x g for 10 minutes and the supernatant was analyzed. for the drug by high pressure liquid chromatography (HPLC) method using the known concentrations of the drug as standard references.

ON44580 Standards:



HPLC analysis of ON44580 standards gave a peak of height of 660 mAU and 94 % area for 1 mg/ml drug concentration.

ON44580 released from transferring nanoparticles:



HPLC analysis of ON044580 released from nanoparticles shows a height of 823 mAU and a 90 % area under the curve. This is equivalent to 1.22 mg/ml. The original sample was diluted three-fold in acetonitrile, hence the preparation contains 3.66 mg/ml of ON044580. A total of 8 mg ON044580 was used in the preparation of the nanoparticles. This translates to approximately 45% encapsulation efficiency. Given the molecular weight of ON044580 (514 daltons) the molar concentration of ON044580 in this preparation is estimated to be 7.12 mM.

Doxorubicin nanoparticles

Similar protocols were employed to make and assess doxorubicin (a known chemotherapeutic with potent anticancer activity) encapsulation in transferring nanoparticles. We found that our preparation of doxorubicin had an encapsulation efficiency of 40% and a molar concentration of 40 µM.

ON044580 loaded transferrin nanoparticles are non-toxic to mice

Having prepared drug containing transferring nanoparticles we tested their toxicity profiles in mice. Female CD-1 mice (25 grams) were injected (IP injections) with a sublethal (24 mg/kg) and lethal dose (64 mg/kg) of ON044580 formulated as nanoparticles. The mice were observed for over 2 weeks with no signs of toxicity or weight loss. However, the injection of these nanoparticles was difficult due to the "stickiness" of the preparations. This could well be due to the size of the nanoparticles (hundereds of nanometer diameter). We are currently working on making finer nanoparticle preparations for greater ease of administration.

Cytotoxicity of ON044580-transferrin nanoparticles

After determining the safety of the nanoparticles in mice we tested their ability to be endocytosed by tumor cells and cause growth inhibition. We carried out cytotoxicity analysis using ON044580 and doxorubicin containing transferring nanoparticles on two cell lines: 32D:*p210*-T315I cells overexpressing the imatinib resistant mutant of BCR-ABL and Ba/F3:V617F-*JAK2* cells that ectopically overexpress the activated JAK-2 mutant.

As can be seen in Figure 9 the nanoparticles of both drugs were excellent inhibitors of tumor cell growth. Against 32D:*p210*-T315I cells both doxorubicin and ON044580 had GI_{50} values of 25µM. However, as can be seen in Figure 10 against Ba/F3:V617F-*JAK2* cells ON044580 had a GI_{50} value of 9 µM as compared to 24 µM of Doxorubicin. This experiment demonstrates that

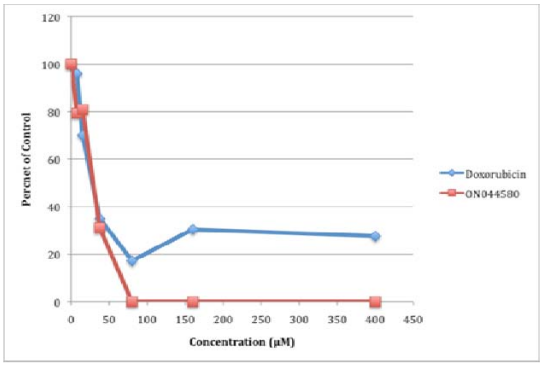


Figure 9: Growth inhibition of 32D:*p210*-T315I cells by ON044580-transferrin nanoparticles. 32D:*p210*-T315I cells were plated at 2.5 x 10^5 cells/ml/well. The cells were treated with increasing amounts of transferrin nanoparticles containing ON044580 and Doxorubicin. The total number of viable cells was determined following trypan blue staining and counting using a haemocytometer 48 hours later. The data is plotted as the percent total viable cells compared to DMSO treated controls.

the nanoparticles are endocytosed by the transferring-transferrin receptor system of actively dividing tumor cells and also that the drug is released from the nanoparticles leading to their cytotoxic effects. However, these GI_{50} values are inferior (350 nM for Ba/F3:V617F-*JAK2* cells) to those achieved by free ON044580 added directly to culture media. This 25 fold lower activity could be a consequence of the aggregation of these relatively large nanoparticles preventing their efficient uptake. Further improvements of nanoparticle preparation are ongoing with the aim to achieve diameters in the sub-100nm range.

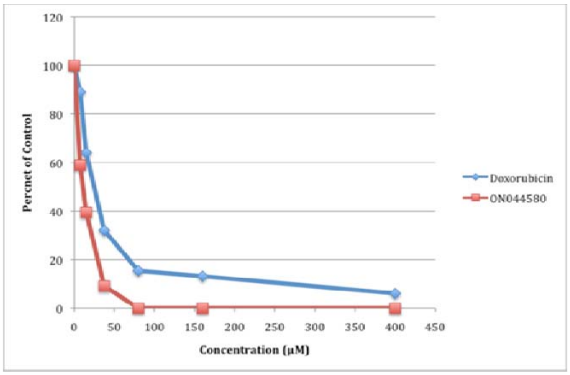


Figure 10: ON044580-loaded transferrin nanoparticles inhibit the growth of Ba/F3:V617F-*JAK2* cells. Ba/F3:V617F-JAK2 cells were plated at 1.0 x 10^5 cells/ml/well. The cells were treated with increasing amounts of transferrin nanoparticles containing ON044580 and Doxorubicin. The total number of viable cells was determined following trypan blue staining and counting using a haemocytometer 48 hours later. The data is plotted as the percent total viable cells compared to DMSO treated controls.

We will test the efficacy of these ON044580 nanoparticles and/or those of its bioactive analogs with better pharmacokinetic properties to treat mouse models of chronic myelogenous leukemia (CML) and myeloproliferative disorders (MPD).

Key research accomplishments:

1. We have developed a bioactive intermediate of ON044580 based on structure activity relationship studies. Further modifications of this intermediate, ON045590 are being developed to obtain an optimized lead molecule with more desirable pharmacokinetic properties.
2. ON045590, is a potent BCR-ABL kinase inihibitor with efficacious cytotoxicity against CML cell lines that express WT or the imatinib resistant T315I mutant form of BCR-ABL. Further, ON045590 is also able to down-regulate BCR-ABL autorphosphorylation and STAT-3 phosphorylation.
3. ON045590 also retained the ability to inhibit kianse activity of the activated V617F mutant form of JAK2 and inhibit the proliferation of a myeloid cell line overexpressing this mutant form of JAK2.
4. We have also developed a transferin nanoparticle strategy for the delivery of ON044580 into tumor cells. Having determined the cytotoxicity of these nanoparticles and their excellent toxicity profile in mice we are working on improving the preparation for treatment of xenograft mouse models of CML and MPD.

Reportable Outcomes

None

Conclusions

1. ON045590, is a potent BCR-ABL kinase inihibitor with efficacious cytotoxicity against CML cell lines that express WT or the imatinib resistant T315I mutant form of BCR-ABL. Further, ON045590 is also able to down-regulate BCR-ABL autorphosphorylation and STAT-3 phosphorylation.

2. ON045590 also retained the ability to inhibit kianse activity of the activated V617F mutant form of JAK2 and inhibit the proliferation of a myeloid cell line overexpressing this mutant form of JAK2.
3. We have also developed a nanoparticle strategy for the delivery of ON044580 into tumor cells.

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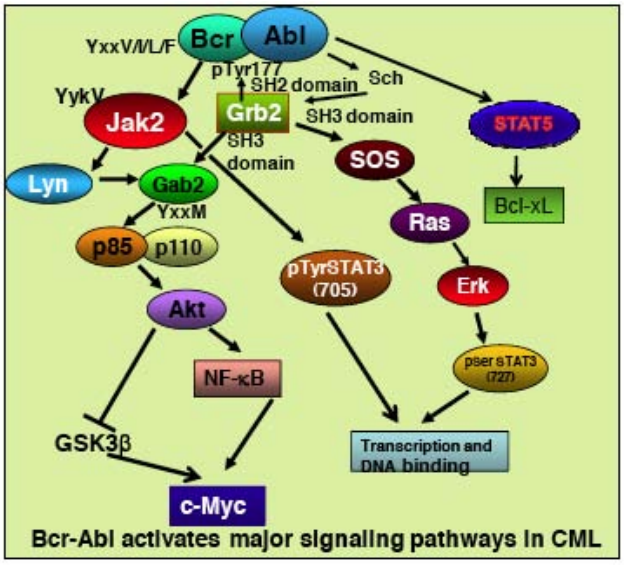
Work Accomplished by Dr. Arlinghaus' Group at the M.D. Anderson Cancer.

INTRODUCTION

During investigation for the basis of drug resistance in CML especially in imatinib, dasatinib drug resistance and also in blast crisis stage of CML where none of the drugs, which were very effective at the early stage of CML, are able to kill the drug-resistant cells (1). We discovered that the concept of target molecule as Bcr-Abl in all stages of CML needs to be reevaluated. From all our studies with the ON-044580 compound we demonstrated that the compound can induce apoptosis in imatinib (IM) sensitive Bcr-Abl+ cells, IM-resistant Bcr-Abl+ cells specifically Bcr-Abl mutant T315I cells, IM-resistant K562-R cells (where Lyn is upregulated) and finally in all blast crisis cells derived from different stages of the CML patients. In last two years report we have demonstrated that ON-044580 compound is a very potent compound and it inhibits a number of signaling pathways that ultimately caused induction of apoptosis in IM-resistant cells. Our subsequent studies showed that in CML cells a large molecular network complex is formed consisting of Bcr-Abl, Jak2, STAT3, HSP90, Akt, GSK3 , Lyn etc. We also showed that Onconova-044580 disrupts the large molecular network complex into a smaller structure in Bcr-Abl+ cells. This work is underway and we are expecting to get the results very soon. In the current report our main focus was to understand the mechanism of action of Onconova 044580 in CML cells. Where it works and why the cells are going rapidly for apoptosis? To get the answers to the above questions we did some experiments using a systematic approach. Our study demonstrates



that Jak2 kinase is a critical target molecule in CML cells (2-4). To establish that Jak2 is an important therapeutic target in the late stages of CML, we have presented several supporting pieces of evidence and we have shown that ON-044580 is a new and potent Jak2- inhibitor. To compare and contrast Jak2 inhibitory effects of the Onconova compound, we have used a specific Jak2-inhibitor TG101209 (TGAY1) as a control. We have already shown the potential of TGAY1 as an anti-leukemic compound, which is effective in all stages of CML (1). The current concept of Bcr-Abl driven signaling net work is presented in the Fig**. shown above.**

Using TGAY1 we have learned that Jak2 is the therapeutic target in CML and that TGAY1 is a potent Jak2 inhibitor, which may be useful for CML therapy. In the current study we have presented the supporting data regarding the mode of action of On044580 and TGAY1 in Bcr-Abl+ cells.

Body

ON-044580 is a Jak2 inhibitor: We observed initially from year 1 study that the compound can induce apoptosis in major IM-resistant cells and blast crisis cells from CML patients (4-6, 7). We suspected that these potent inhibitory effects may be due to inhibition of Jak2 in the Bcr-Abl driven signaling pathway. To establish our hypothesis that Jak2 is a critical regulator of Bcr-Abl signaling, we used a specific Jak2 inhibitor (8) (TargeGen Inc. TG101209, TGAY1) to compare and contrast the Jak2 inhibitory effects with those of ON-044580 in Bcr-Abl+ cells. We concluded that several of the effects that we observed in Bcr-Abl + cells treated with ON-044580 are due to Jak2 inhibitory effects. Let us explore the basis of our hypothesis step by step.

Stability of Bcr-Abl depends on Jak2, and inhibition of Jak2 either by its inhibitor TGAY1 or by On-044580 induced disappearance of Bcr-Abl.

(a) Jak2 inhibition caused a rapid removal of Bcr-Abl from the cell lysate. Incubation of Bcr-Abl+ 32Dp210 cells with On044580 for 16h with different doses showed that pJak2 was reduced in a dose dependent manner starting from 1 to 10μM, doses used for apoptosis experiments. Surprisingly we also observed that under that condition, Bcr-Abl disappeared in a dose response manner starting from 2.5μM (data not shown). To

examine the time period of the interaction of On044580 with the cells, we incubated the cells with 10 μM of the reagent for different time period starting from 15 min to 120 min. After 60 min Bcr-Abl level was reduced (**Fig 1**). In order to compare the property with Jak2 inhibitors, we incubated Bcr-Abl+ cells with TGAY1 under identical conditions and we observed that Bcr-Abl was reduced by Jak2 inhibition (Fig 3). We explored the basis of Bcr-Abl disappearance.

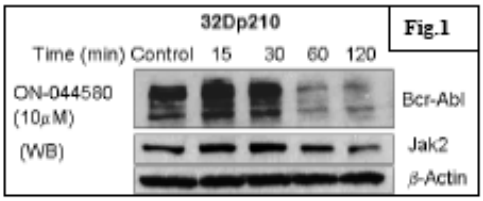


Fig.1 Incubation of Bcr-Abl+ cells with ON-044580 (10μM) rapidly reduced Bcr-Abl level as detected by Western blotting using antibody of Bcr-Abl(8e9). Although pJak2 level was reduced (not shown in the figure) Jak2 level was slowly altered. (**Fig4**).

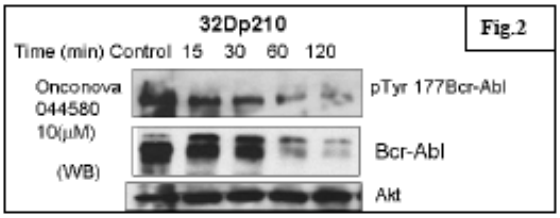
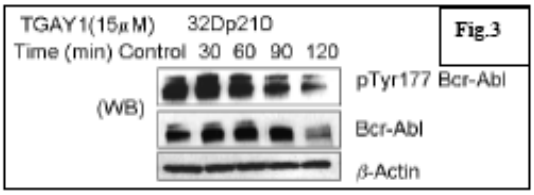
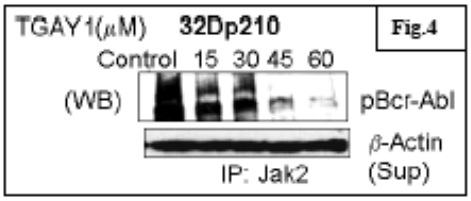


Fig.2 Incubation of Bcr-Abl+ cells with ON-044580 reduced pTyr177Bcr-Abl as well as Bcr-Abl level as detected by Western blotting probed with respective antibodies. Akt level was not reduced with in this time frame.





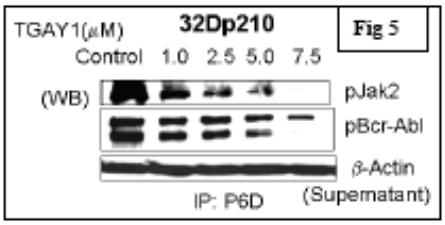


Fig. 3-5 Incubation of Bcr-Abl+ cells with Jak2 specific inhibitor (TGAY1) reduced Bcr-Abl, pBcr-Abl, and Tyr177Bcr-Abl in time dependant (Fig.6-7) and dose dependant manner (Fig.8). For Fig7 Jak2 was immunoprecipitated and pBcr-Abl was detected by Western blotting using pTyr antibody. For pJak2 detection, Bcr-Abl was immunoprecipitated by Bcr-Abl antibody (P6D).

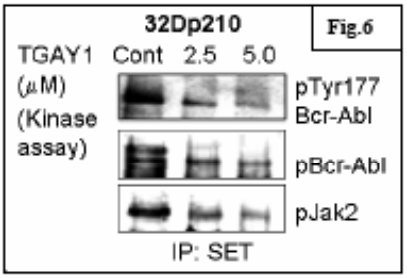


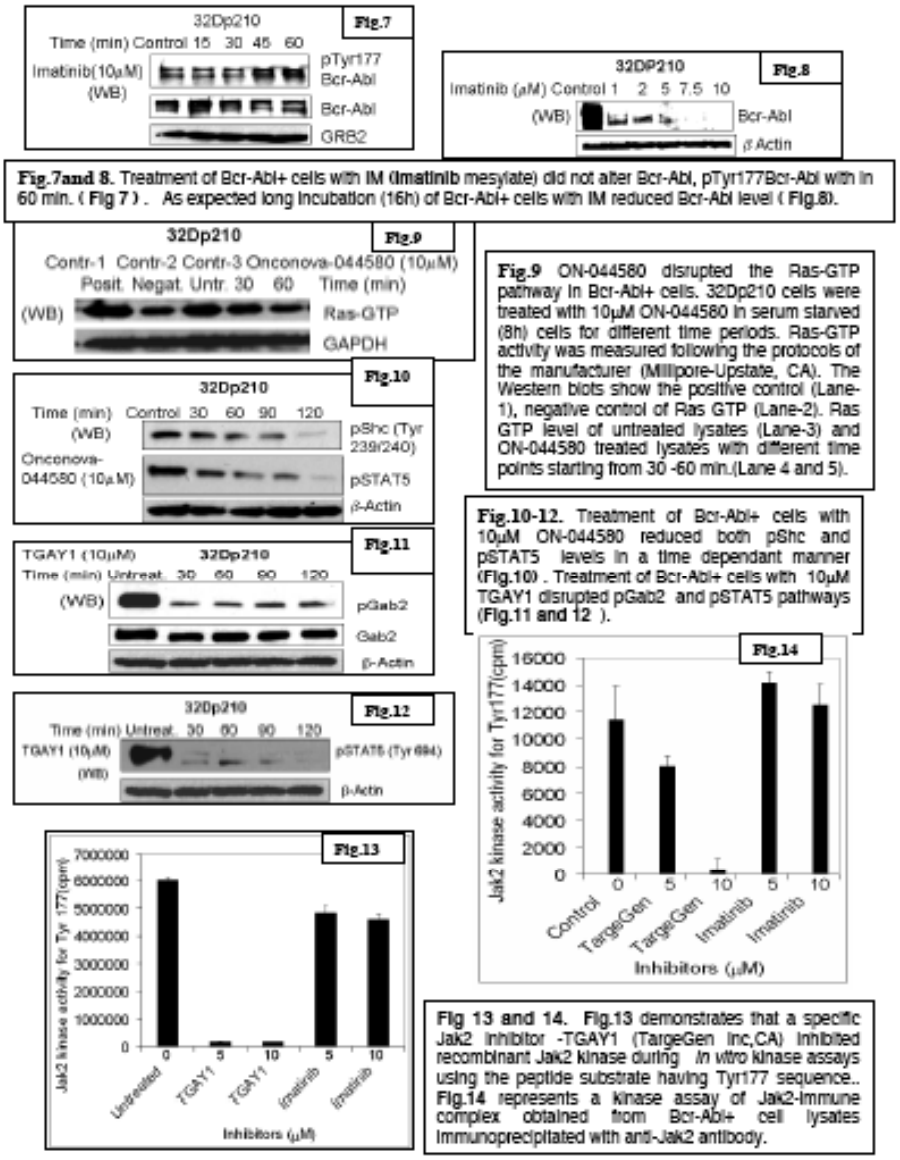
Fig. 6. In an in vitro kinase assay TargeGen Jak2 inhibitor TGAY1 inhibited pJak2, pBcr-Abl and pTyr177 Bcr-Abl in the Bcr-Abl+ cell lysates immunoprecipitated with SET. After kinase assay, Western blotting was carried out and the proteins were detected by respective antibodies. pBcr-Abl and pJak2 were detected by anti-pTyr antibody.

(b) <u>On044580 inhibited phosphorylation of Tyr177 Bcr-Abl:</u> In Bcr-Abl there are about a number of tyrosine phosphorylation sites involved in several process. Bcr sequences are phosphorylated at Tyr177, Tyr 360, and Tyr283. Tyr 360 and Tyr 283 are responsible for

catalytic activity (auto and transkinase activities) of Bcr. We and others have shown that Bcr-Abl is phosphorylated on tyrosine 177 (9). Note that after Jak2 inhibition, total Bcr-Abl protein levels also rapidly disappear but at a slower rate than pTyr 177/pTyr 360 and total pTyr residues of Bcr-Abl (**Fig. 3**). Both these tyrosine residues have consensus sequences for Jak2 dependent phosphorylation (YxxV/I/L/F) (10). This event leads to binding of Bcr-Abl to Grb2 and activation of the SOS-Ras pathway (4), and activation of the PI-3 kinase through the Grb2-Gab2 pathway. We anticipated that instability of Bcr-Abl may be due to inhibition of pTyr177. We surprisingly demonstrated that indeed, pTyr177 was reduced in Bcr-Abl+ cells in a time dependent manner when incubated Bcr-Abl+ cells with On044580 (**Fig. 2**). We also observed the similar effects in TGAY1 treated cells (Fig. 3). Total pBcr-Abl level was also reduced in Jak2 inhibitor treated cells (Fig.3-5). In an in vitro kinase assay SET was used to immunoprecipitate Bcr-Abl and pJak2. We demonstrated that addition of Jak2 inhibitors to the kinase assay reduced pJak2 level, pTyr177 and but not the total Bcr-Abl level (**Fig. 6**). These results indicate that inhibition of Jak2 for just 15 min caused removal of phosphates from tyrosine 177, suggesting that active Jak2 is continuously required to phosphorylate these two sites, and possibly others sites in Bcr-Abl. Similarly, TGAY1 also inhibited phosphorylation of Tyr 177 of Bcr-Abl under these conditions (Fig.3) and in kinase assays conducted on anti-SET IPs (Fig. 6).

imatinib mesylate (IM) did not rapidly inhibit tyrosine phosphorylation of Bcr-Abl on Tyr 177, nor did IM cause rapid loss of Bcr-Abl from the cell lysate (Fig. 7). However, treatment of Bcr-Abl+ cells with IM for much longer times decreases Bcr-Abl protein levels (Fig. 8) but at a much later time (more than 6 h).

On044580 disrupted Bcr-Abl driven signaling pathways: Autophosphorylation of Bcr-Abl at Tyr 177 makes a docking site for scaffolding protein Grb2 through its SH2 domain. This leads to phosphorylation of Gab2 due to a Jak2/Lyn kinase pathway (unpublished) and recruitment of Gab2 to Grb2 through its SH3 domain. One site of Gab2 is bound to a regulatory subunit of PI3 kinase through which Akt is eventually phosphorylated (11, 12). Another site of SH3 domain of Grb2 is connected to SOS molecule through which Ras is connected with Ras –Raf-Erk pathway (15). These



pathways are critical for cell proliferation, survival and transformation. Thus, treatment of Bcr-Abl+ cells with On044580 is expected to disrupt both of these signaling pathways through reduction pTyr 177 of Bcr-Abl (our hypothesis). In the following experiments we have presented evidence in support of this hypothesis.

(a) *Ras-GTP pathway is disrupted by On044580*- We have measured Ras-GTP levels after treatment of Bcr-Abl+ cells with On044580 in serum starved cells (8h). After that we analyzed Ras-GTP following the manufacturer protocol following their kit (Millipore-Upstate Company). Figure 9 shows that with respect to untreated cells, Ras-GTP (15) level was reduced within 60 min, indicating that Ras pathway is being strongly inhibited by On044580 (Fig.9)..

(b) *pShc (Tyr 239/240) and pSTAT5 (Tyr694) (16) were reduced* **–** To support the hypothesis further that On044580 inhibits signaling pathways in Bcr-Abl+ cells, we measured pShc and pSTAT5 level in the Bcr-Abl+ cells treated with On044580. We observed that both pShc and pSTAT5 levels were reduced in a time dependant manner and the signals completely disappeared at 120 min after the incubation of the cells with On044580 (Fig.10). To confirm that this property of disruption of Bcr-Abl signaling pathways was contributed by Jak2 inhibition, we treated the cells with TGAY1 and under identical conditions and we measured pSTAT5 and pGab2 (**Fig. 11 and 12**). We observed the pSTAT5 level was reduced in On044580 treated cells indicating that Jak2 kinase inhibitory effect of On044580 also disrupted these additional Bcr-Abl signaling pathways. We believe these effects of On044580 on pTyr STAT5 are due to Jak2 inhibition, which causes the severe reduction of Bcr-Abl. Thus, Bcr-Abl is not available to activate STAT5. The effects of On044580 on pShc are more complex, and needs further experimentation. We suggest that Jak2 also phosphorylates the YvnV sequence of Shc, and that Jak2 inhibition then prevents its phosphorylation at this Tyr site, thus preventing Grb2 binding to pTyr Shc. This leads to disruption of the Bcr-Abl/Shc/Grb2 activation of the Ras and PI-3 kinase pathways as shown above for pTyr 177 (TvnV) of Bcr-Abl.

In vitro Jak2 kinase assays with recombinant Jak2 show that Jak2 inhibitors TGAY1 and ON-044580 block phosphorylation of Tyr 177 Bcr peptide. As demonstrated earlier, pTyr 177 formation was rapidly and strongly inhibited by Jak2 inhibition by TGAY1 and also by On044580. We determined whether the Jak2 kinase directly phosphorylates Tyr177 of Bcr-Abl. To examine this question we used both Jak2 immune complexes obtained from Bcr-Abl+ cell immunoprecipitated with Jak2-coupled agarose beads (Santa Cruz, CA) and recombinant Jak2 kinase (JH1 domain) (Invitrogen, CA). We used a synthetic Bcr peptide containing the Tyr177 sequence of Bcr-Abl, which was synthesized by Bachem Co, The sequence is as follows: H-Ala-Glu-Lys-Pro-Phe-Tyr-Val-Asn-Val-Glu-Phe-His-His-Glu-Lys-Lys-Lys-OH (Molecular mass 2130.5). The three C-terminal Lys residues are added to allow binding of the peptide substrate to Whatman filters (see ref by Lin et al). The kinase assay of Jak2 was done as described

(19) in presence of either Jak2 inhibitor TGAY1 or or Abl kinase inhibitor Imatinib. We observed that the Jak2 inhibitor strongly inhibited catalytic activity of the enzyme whereas Imatinib failed to inhibit (Fig.13 and 14). Under identical conditions we measured Jak2 kinase activity using recombinant Jak2 kinase and same peptide substrate in presence and absence of On044580. We observed that Jak2 kinase activity was reduced in presence of Ono44580 in a dose-response manner. It suggests that Jak2 inhibitory effects of Onconova compound is very inhibitory but is slightly less potent than TGAY1.

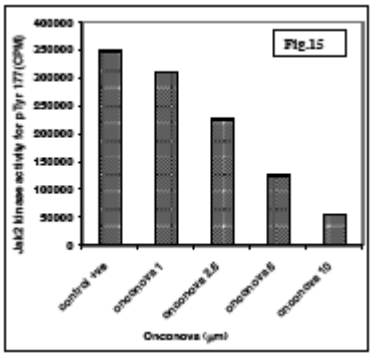


Fig.15. In vitro Jak2 kinase assay with recombinant Jak2 protein (JH1 domain) in presence of Onconova -044580(Fig15). The peptide substrate was synthesized by Bachem Co and the sequence is as follows: H-Ala-Glu-Lys-Pro-Phe-Tyr-Val-Asn-Val-Glu-Phe-His-His-Glu-Lys-Lys-Lys-OH (Molecular mass 2130.5). Recombinant Jak2 (JH1 domain) was preincubated with different amount of **Onconova-044580** or Jak2 inhibitor for 10 min. The assay was carried out in presence of 32P gamma ATP, cold ATP, Mg++,Mn++, DTT at 30)C for 15 min and the reaction product was equally dropped on the Whatman filter paper disk (2.3 mm diameter) which was immersed in counting fluid and counted in a Liquid Scintillation counter.

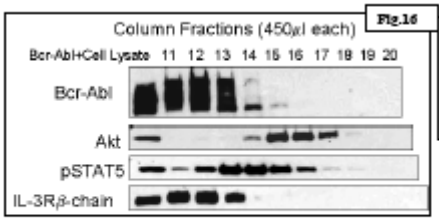


Fig. 16. Bcr-Abl+ cells fractionated in column chromatography demonstrated that Bcr-Abl was eluted with the same fraction of IL-3b chain receptor and Akt and STAT5 were derived from separate network complex.

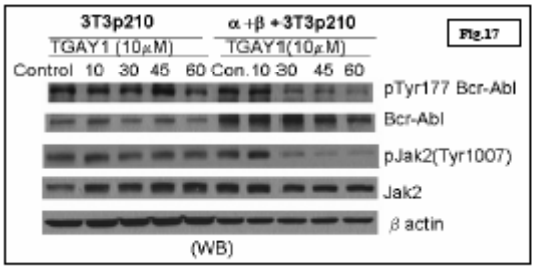


Fig. 17. Functionally active pJak2 is expressed in triple positive (right side) but not in Bcr-Abl+ transfected fibroblast cells (left panel) and the level of pJak2 is reduced by Jak2 inhibitor -TGAY1 in triple positive cells only resulting in reduction of the levels of pTyr177Bcr-Abl, Bcr-Abl (right panel).

<u>The IL-3 receptor is associated with Bcr-Abl leading to activation of Jak2 but pSTAT5 is not connected to the Bcr-Abl/Jak2 /IL-3 receptor pathway (Tao et al ref)</u>: In our previous report we have shown that Bcr-Abl forms a network complex with Jak2, Gab2, PI-3 kinase and HSP90. Other Bcr-Abl network complexes may exist that contain STAT3, STAT5 and AKT (4). . We have recently demonstrated that Bcr-Abl is also physically associated with IL-3 receptors, pSTAT5 in Bcr-Abl+ cells. We were able to detect this complex (es) from the 32Dp210 cell lysates by fractionation on a Superose TM 6 column. The proteins were detected at a size between 6 and 2 million Daltons (**Fig.16**). Fractions were assayed by Western Blotting with the various antibodies as indicated. The work is in progress to compare the effects of TGAY1 and On044580 on the disruption of Bcr-Abl net work complex in Bcr-

Abl+ cells. Preliminary experiments show that On044580 disrupts the Bcr-Abl/Jak2 network (not shown).

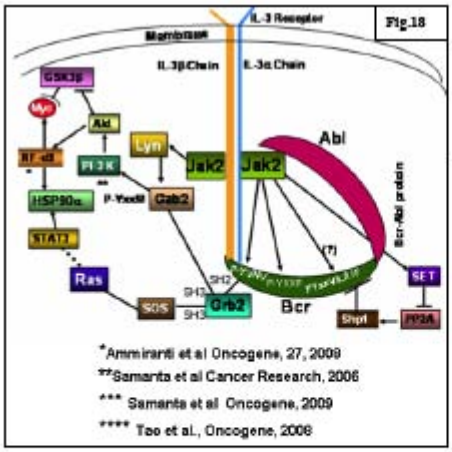
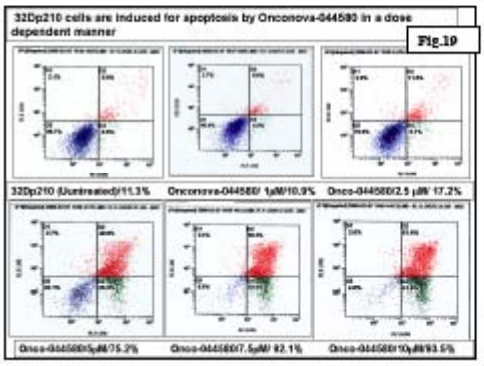
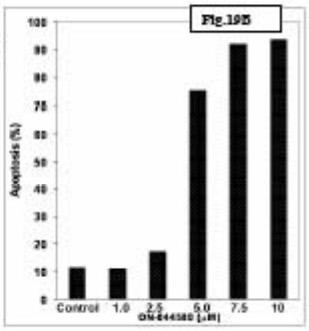


Fig.18. Model of the role of Jak2 in regulating Bcr-Abl signaling. Note that Jak2 is associated with the C-terminus of Abl within Bcr-Abl. (Ref. 3) Based on experiments shown here using the Bcr-Abl IL-3 receptor system, we have devised the model shown here.





Jak2 phosphorylation of Tyr 177 is stimulated by the presence of the IL-3 receptor. Recently Tao et al (17,18)have demonstrated that for activation of Jak2 both chains of IL-3 receptor are required, which was demonstrated in a Bcr-Abl fibroblast - NIH3T3 cell transfection system (5). The NIH 3T3 cell system also gave useful information, as cells expressing Bcr-Abl and the IL-3 receptor had activated Jak2 and treatment with 10 μM TGAY1 caused rapid disappearance of pTyr 177 but not in Bcr-Abl+ NIH 3T3 cells lacking the receptor (**Fig. 17**). That activated Jak2 is critical for phosphorylation of Tyr 177 is confirmed by the relative lack of Jak2 activation in cells lacking the IL-3 receptor but expressing Bcr-Abl (**Fig. 17**). A model depicting the involvement of IL-3 receptor for the activation of pJak2 is presented in **Fig.18**. Work is in progress to determine the effects of pJak2 activation and pTyr177 Bcr-Abl expression in presence of On044580 in the Bcr-Abl this system (18).

Induction of apoptosis in IM-sensitive and resistant cells by On044580. We examined the ability of On044580 to induce apoptosis in various BCR-ABL+ cells at 1-10 μM for the compound for 48h. MTT assays showed that On044580 strongly reduced viability of

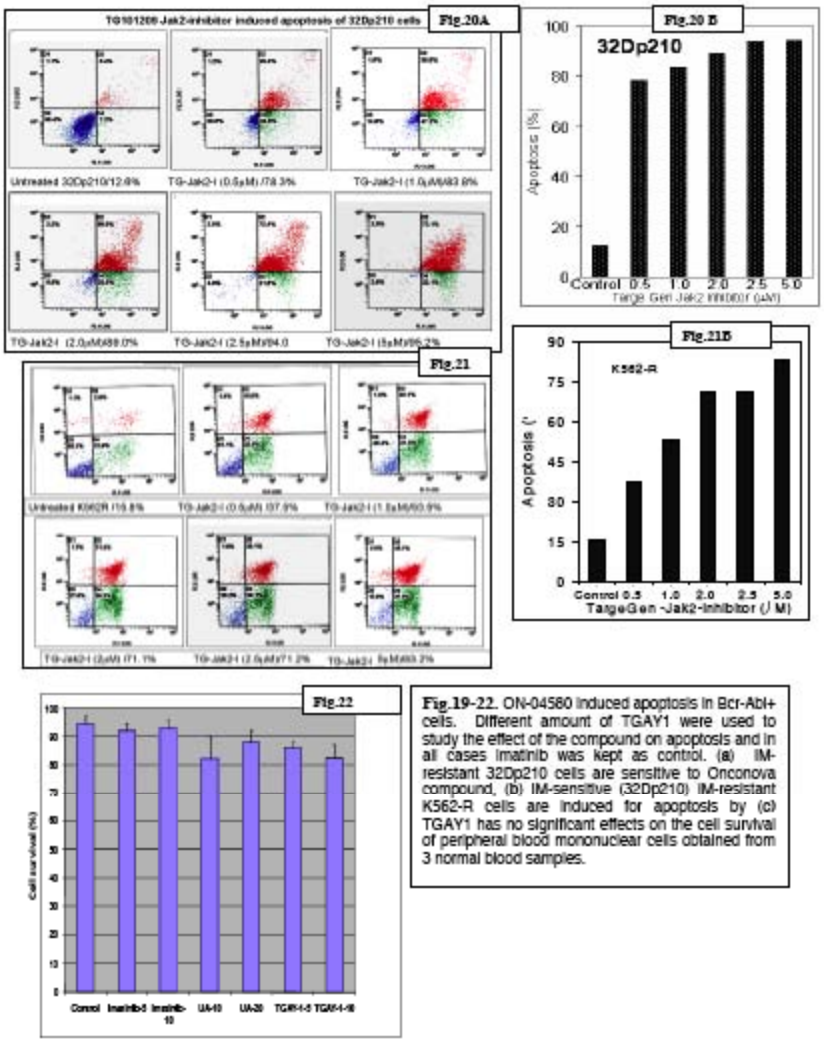


Fig.19-22. ON-04580 induced apoptosis in Bcr-Abl+ cells. Different amount of TGAY1 were used to study the effect of the compound on apoptosis and in all cases Imatinib was kept as control. (a) IM-resistant 32Dp210 cells are sensitive to Onconova compound, (b) IM-sensitive (32Dp210) IM-resistant K562-R cells are induced for apoptosis by (c) TGAY1 has no significant effects on the cell survival of peripheral blood mononuclear cells obtained from 3 normal blood samples.

K562 cells and IM-resistant K562-R cells within 72 h (IC-50 at about 2.5 μM), which we have reported earlier. Analysis for apoptosis induction showed that 32Dp210 cells **(Fig.19)** underwent high levels of apoptosis with 5-10 μM On044580 (IC-50 about 2.5 μM). (**Fig.19A and B**). Similarly, BaF3 T315I Bcr-Abl+ cells and K562-R cells showed strong induction of apoptosis by the On044580 as we reported earlier. Here we show that this induction of apoptosis was due to Jak2 inhibitory effects as we demonstrate that TGAY1 which can induce apoptosis of Bcr-Abl+ IM sensitive and resist ant cells (**Fig 20A and B and Fig. 21A and B). Of interest** we showed that viability of peripheral mononuclear cells derived from three normal persons was not affected by either TGAY1 or by Ursolic acid compared to untreated cells **(Fig.22)**

Key Research Accomplishments

1) *Bcr-Abl stability is under control of Jak2 kinase*

(2) Jak2 controls phosphorylation of Ty177 of Bcr-Abl;, therefore, SOS-Ras-RAF pathway and PI3 kinase-Akt pathways are under control of Jak2in Bcr-Abl+ cells

(3) Jak2 Phosphorylates Tyr705 STAT3

(4) Jak2 regulates NF-kB activation (Samanta et al. Cancer Research)

(5) Jak2 regulates HSP90 synthesis

(6)We found that Jak2 has a critical role in the formation and stabilization of Bcr-Abl-Jak2-HSP90 network complex.

(7)Lyn kinase, which is highly expressed during IM resistant (K562-R cells is controlled by Jak2 (Samanta et al Oncogene **28**, 1669-1681 (2009).

(8) SET activation is under control of Jak2 not Bcr-Abl

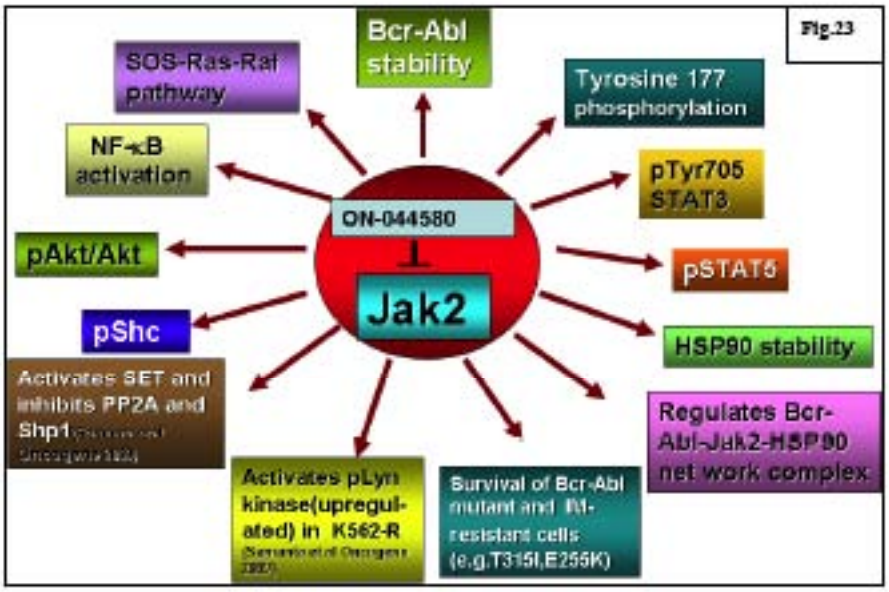


Fig. 23. Model shows that Jak2 regulates all the vital and critical functions of Bcr-Abl – driven signaling pathways in CML. Inhibition of Jak2 functions by ON-044580 or by specific Jak2 inhibitor TGAY1 inhibited Jak2 kinase and induced for apoptosis in IM sensitive and resistant cells including cells from blast crisis patients .

Reportable Outcomes

None

<u>Coclusions</u>

1) Bcr-Abl stability is under control of Jak2 kinase

(2) Jak2 controls phosphorylation of Ty177 of Bcr-Abl;, therefore, SOS-Ras-RAF pathway and PI3 kinase-Akt pathways are under control of Jak2in Bcr-Abl+ cells

(3) Jak2 Phosphorylates Tyr705 STAT3

(4) Jak2 regulates NF-kB activation

(5) Jak2 regulates HSP90 synthesis

(6)We found that Jak2 has a critical role in the formation and stabilization of Bcr-Abl-Jak2-HSP90 network complex.

(7)Lyn kinase, which is highly expressed during IM resistance (K562-R cells) is controlled by Jak2

(8) SET activation is under control of Jak2 not Bcr-Abl

Future Plan of Work with no cost extension:

1) We plan to complete the identification and characterization of Bcr-Abl net work complex (es) through in vitro column chromatography combined with immunoprecipitation and mass spec analyses.

2) To examine the effects of On044580 and Jak2 inhibitor-TGAY1 on the Bcr-Abl network complex in Bcr-Abl + cells. We will follow up the dissociation of individual molecules from HSP90 complex.

3) We want to examine the involvement of Jak2 in phosphorylation of Tyr177 Bcr-Abl by knocking down Jak2 by transient transfection of Jak2 siRNA or by a stable transduction of Jak2 shRNA in Bcr-Abl+ cells combined with rescue of the Jak2 knockdown cells with expression of excess Jak2.

4) Finally we would like to examine how stability of HSP90 is controlled by Jak2 in CML cells and the overall impact of cell signaling connected to HSP90 in On044580 treated Bcr-Abl+ CML cells.

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Work Accomplished by Onconova Therapeutics

Body

1. Onconova chemists synthesized several analogues of ON044580, including ON044590 to screen for BCR-ABL inhibitory activity by Dr. Reddy's group.

2. Onconova scientists worked in collaboration with Dr. Reddy's group to develop the Caco cell assay and carried out HPLC analyses to determine encapsulation efficiency of ON044580 loaded transferrin nanoparticles.

3. Onconova developed methods for large scale synthesis of ON044580 and ON044590 and supplied gram quantities of these compounds to Drs. Reddy and Arlinghaus for their studies.

4. Onconova scientists have initiated studies to determine the oral bio-availability of ON044590 in mouse model systems.

Key Research Accomplishments

Please see Dr. Reddy's and Dr. Arlinghaus' reports.

Reportable Outcomes

None

Conclusions

Please see Drs. Reddy and Arlinghaus' conclusions.